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Two amino acid mutations in an anti-human CD3 single chain Fv antibody fragment that affect the yield on bacterial secretion but not the affinity

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Recombinant antibody fragments directed against cell surface antigens have facilitated the development of novel therapeutic agents. As a first step in the creation of cytotoxic immunoconjugates, we constructed a single-chain Fv fragment derived from the murine hybridoma OKT3, that recognizes an epitope on the ε-subunit of the human CD3 complex. Two amino acid residues were identified that are critical for the high level production of this scFv in *Escherichia coli*. First, the substitution of glutamic acid encoded by a PCR primer at position 6 of V_H framework 1 by glutamine led to a more than a 30-fold increase in the production of soluble scFv. Second, the substitution of cysteine by a serine in the middle of CDR-H3 additionally doubled the yield of soluble antibody fragment without any adverse effect on its affinity for the CD3 antigen. The double mutant scFv (Q,S) proved to be very stable *in vitro*: no loss of activity was observed after storage for 1 month at 4°C, while the activity of scFv containing a cysteine residue in CDR-H3 decreased by more than half. The results of production yield, affinity, stability measurements and analysis of three-dimensional models of the structure suggest that the sixth amino acid influences the correct folding of the V_H domain, presumably by affecting a folding intermediate, but has no effect on antigen binding.

Keywords: affinity/anti-human CD3/bacterial expression/single-chain Fv/solubility

Introduction

In recent years, the use of genetic engineering techniques has stimulated the development of antibody-like molecules for therapeutic and diagnostic uses (Winter and Milstein, 1991). Unlike glycosylated whole antibodies, fragments such as Fab and Fv can be easily produced in bacterial cells as functional antigen binding molecules (Better *et al.*, 1988; Skerra and Plückthun, 1988). To stabilize the association of the recombinant V_H and V_L domains, they have been linked in a single-chain Fv (scFv) construct with a short peptide that connects the carboxy terminus of one domain and the amino terminus of the other (Bird *et al.*, 1988; Huston *et al.*, 1988). In comparison with the much larger Fab, $F(ab)_2$ and IgG forms of monoclonal antibody from which they are derived, scFvs have more rapid blood clearance and better tumor penetration (Milenic *et al.*, 1991; Yokota *et al.*, 1992; Adams *et al.*, 1993). ScFvs therefore represent potentially highly useful molecules for the targeted delivery of drugs, toxins or radionuclides to a tumor site.

The efficient expression of active antibody fragments in bacteria is clearly of great technological importance. However, as with the expression of some other heterologous proteins in *Escherichia coli*, the yield of functional product for some antibody fragments can be very low. Sometimes, PCR primer-induced errors can lead to the expression of non-reactive antibody fragments (McCartney *et al.*, 1995). Poor expression may also arise from differences in the translation machinery and folding pathways of eukaryotic and bacterial cells. For example, some nucleotide sequences encoding antibody variable regions were expressed as functional proteins in eukaryotic host cells but were unable to express a product in bacteria (Duenas *et al.*, 1995). Limiting factors for the efficient production of secreted antibody fragments in *E.coli* appear to be translocation to the periplasm (Ayala *et al.*, 1995) and folding in the periplasmic space (Knappik and Plückthun, 1995).

OKT3 is a murine monoclonal antibody (mAb) that recognizes an epitope on the ε-subunit of the human CD3 complex (Kung *et al.*, 1979; Van Wauwe *et al.*, 1980; Transy *et al.*, 1989). It has significant clinical utility. OKT3 has been widely used to suppress T cells and thereby prevent the rejection of transplants (Thistlethwaite *et al.*, 1984; Woodle *et al.*, 1991). Conversely, T cell activation and proliferation induced by OKT3 have been exploited to expand effector cells *ex vivo* for adoptive cancer immunotherapy (Yannelly *et al.*, 1990). As well as being used alone, the OKT3 mAb has been used as a component of bispecific antibodies to retarget cytotoxic T lymphocytes against tumor cells (Nitta *et al.*, 1990; Bohlen *et al.*, 1993) or virus infected cells (Sanna *et al.*, 1995). Recently, humanized versions of the OKT3 mAb have been expressed in COS cells (Woodle *et al.*, 1992; Adair *et al.*, 1994).

In this paper, we present the first example of the expression of an OKT3 derived scFv in *E.coli*. As part of the anti-CD3 scFv construction process, the PCR amplified OKT3 V_H gene was modified to improve its *in vivo* folding. Here we analyze the effect of two amino acid residues in the variable heavy chain domain on the yield, affinity and stability *in vitro* of anti-CD3 scFv.

Materials and methods

E.coli strains, plasmids and cell lines

E.coli K12 strain XL1-Blue (Stratagene, La Jolla, CA) was used as the cloning and expression host. For cloning, sequencing hybridoma-derived immunoglobulin variable regions and site-specific mutagenesis, pCR-Script SK(+) (Stratagene) was used. The scFv gene was assembled and expressed either in the plasmid pOPE51 (Kipriyanov *et al.*, 1994) or in pHOG21 (Kipriyanov *et al.*, 1996b). The hybridoma OKT3 producing a monoclonal antibody (IgG2a) against the CD3 human T cell antigen has been described previously (Kung *et al.*, 1979; Van

EXHIBIT A

Wauwe *et al.*, 1980). The human CD3-positive acute T cell leukemia cell line Jurkat and a CD3-negative B cell line JOK-1 were used for flow cytometry.

Cloning of the variable regions

Isolation of mRNA from freshly subcloned hybridoma OKT3 cells and cDNA synthesis were performed as previously described (Dübel *et al.*, 1994). DNA coding for the light chain variable domain was amplified by PCR using the primers Bi5 and Bi8 that hybridize to the amino terminal portion of the κ chain constant domain and the framework 1 (FR1) region of the κ chain variable domain (Dübel *et al.*, 1994). For the amplification of DNA coding for the heavy chain variable domain, the primer Bi4 that hybridizes to the amino terminal portion of the γ chain constant 1 domain (Dübel *et al.*, 1994) and Bi3f that hybridizes to the FR1 region of the heavy chain (Gotter *et al.*, 1995; Kipriyanov *et al.*, 1996b) were used. The 50 µl reaction mixture contained 10 pmol of each primer and 50 ng of hybridoma cDNA, 100 µM each of dNTP, 1×Vent-buffer (Boeringer Mannheim, Mannheim, Germany), 5 µg BSA and 1 U *Vent* DNA polymerase. 30 cycles of 1 min at 95°C, 1 min at 55°C and 2 min at 75°C were carried out in a thermocycler. The amplified DNA was purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and blunt end ligated into an *Srf*I digested pCR-Script SK(+) (Stratagene) for dideoxy sequencing (Sanger *et al.*, 1977) and site-specific mutagenesis.

Construction of plasmids encoding scFv

The linker used in this study was a 17 amino acid tag-linker that includes a tubulin epitope recognized by mAb YOL1/34 (Breitling *et al.*, 1991). DNA coding for the variable domains of OKT3 was inserted into pOPE51 (Kipriyanov *et al.*, 1994) in two cloning steps using *Nco*I/*Hin*dIII for the heavy-chain DNA and *Eco*RV/*Bam*HI for the light-chain DNA. The whole scFv gene was recloned in pHOG21 (Kipriyanov *et al.*, 1996b) as a *Nco*I/*Bam*HI DNA fragment.

Construction of anti-CD3 mutants

Mutations were generated in the V_H domain derived from OKT3 by site-specific mutagenesis according to Kunkel *et al.* (1987). The amino acid substitution of Cys at position H100A by Ser and of Glu at position H6 by Gln was achieved using either primer SK1 5'-GTAGTCAAGGCTGTAATGATCATC or SK2 5'-GCCCCAGACTGCTGCAGCTGCAC or both.

E.coli expression and purification of scFv fragments

XL1-Blue *E.coli* cells (Stratagene) transformed with the scFv expression plasmid pHOG21 were grown overnight in 2×YT medium with 50 µg/ml ampicillin and 100 mM glucose ($2\times YT_{GA}$) at 37°C. Dilutions (1:50) of the overnight cultures in $2\times YT_{GA}$ were grown as flask cultures at 37°C with shaking at 200 r.p.m. When cultures reached OD_{600} = 0.8, bacteria were pelleted by centrifugation at 1500 *g* for 10 min and 20°C and resuspended in the same volume of fresh 2×YT medium containing 50 µg/ml ampicillin and 0.4 M sucrose. IPTG was added to a final concentration of 0.1 mM and growth was continued at room temperature (20–22°C) for 20 h. The cells were harvested by centrifugation at 5000 *g* for 10 min and 4°C. The culture supernatant was retained and kept on ice. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 5% of the initial volume of ice-cold 50 mM Tris–HCl, 20% sucrose, 1 mM EDTA, pH 8.0. After a 1 h incubation on ice with occasional stirring, the spheroplasts were centrifuged at 30 000 *g* for 30 min and 4°C leaving the soluble periplasmic extract as the supernatant and spheroplasts plus the insoluble periplasmic material as the pellet. The culture supernatant and the soluble periplasmic extract were combined, clarified by additional centrifugation (30 000 *g*, 4°C, 40 min) and passed first through a glass filter of pore size 10–16 µm and then through a Membrex TF filter of pore size 0.2 µm (MembraPure, Lörzweiler, Germany). The volume was reduced 10-fold by concentration with Amicon YM 10 membranes (Amicon, Witten, Germany). The concentrated supernatant was clarified by centrifugation and thoroughly dialyzed against 50 mM Tris–HCl, 1 M NaCl, pH 7.0 at 4°C. Immobilized metal affinity chromatography (IMAC) was performed at 4°C using a 5 ml column of Chelating Sepharose (Pharmacia) charged with Ni^{2+} and equilibrated with 50 mM Tris–HCl, 1 M NaCl, pH 7.0 (start buffer). The sample was loaded by passing the sample over the column. It was then washed with 20 column volumes of start buffer followed by start buffer containing 50 mM imidazole until the absorbance (280 nm) of the effluent was minimal (about 30 column volumes). Absorbed material was eluted with 50 mM Tris–HCl, 1 M NaCl, 250 mM imidazole, pH 7.0. After buffer exchange to 50 mM MES, pH 6.0, the protein was further purified on a Mono S ion-exchange column (Pharmacia). The purified scFv was dialyzed into PBS (15 mM sodium phosphate, 0.15 M NaCl, pH 7.4). For long-term storage, scFv were frozen in presence of BSA (final concentration 10 mg/ml) and kept at –80°C, as recommended (Kipriyanov *et al.*, 1995).

Isolation of scFv from inclusion bodies of bacteria transformed with plasmid pOPE51 was performed essentially as described previously (Kipriyanov *et al.*, 1996a).

SDS–PAGE and Western blot analysis

SDS–PAGE was carried out according to Laemmli (1970) under reducing conditions. Immunoblot analysis using anti *c-myc* mouse mAb 9E10 (Cambridge Research Biochemicals, Cambridge, UK) was performed as described previously (Kipriyanov *et al.*, 1994).

Analyses of scFv stability

For stability analyses, scFv preparations were stored at 4°C at a concentration 50 µg/ml in PBS for 1 month. The activities of samples after storage were determined by flow cytometry.

Flow cytometry

We incubated 5×10^5 $CD3^+$ Jurkat or $CD3^-$ JOK-1 cells in 50 µl RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) and 0.1% sodium azide (referred to as complete medium) with 100 µl of a sample containing scFv for 45 min on ice. After washing with complete medium, the cells were incubated with 100 µl of 10 µg/ml anti *c-myc* mAb 9E10 (ICI Biochemicals) in the same buffer for 45 min on ice. After a second washing cycle, the cells were incubated with 100 µl of FITC-labeled goat anti-mouse IgG (Gibco BRL) under the same conditions as before. The cells were then washed again and resuspended in 100 µl of a 1 µg/ml solution of propidium iodide (Sigma, Deisenhofen, Germany) in complete medium to exclude dead cells. The relative fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Measurement of binding affinity

Affinities were derived either from the FACScan analysis of direct binding of scFv to Jurkat cells as described by Chamow *et al.* (1994) or from a competitive inhibition assay. In the

latter case, increasing concentrations of scFv were added to a subsaturating concentration of FITC-labeled mAb OKT3 (7.4 nM) and were incubated with Jurkat cells as described above. Fluorescence intensities of stained cells were measured as described above. Binding affinities were calculated according to the following equation derived from that of Schodin and Kranz (1993):

$$K_{a(I)} = (1 + [\text{FITC-OKT3}] \times K_{a(OKT3)})/IC_{50}$$

where I is the unlabeled inhibitor (scFv), [FITC-OKT3] is the concentration of FITC-labeled mAb OKT3, $K_{a(OKT3)}$ is the binding affinity of mAb OKT3 (1.2×10^9 M^{-1}; Adair *et al.*, 1994) and IC_{50} is the concentration of inhibitor that yields 50% inhibition of binding.

Determination of the yield of soluble antibody fragments

The expression levels of soluble scFv fragments were determined in cleared culture medium and in crude periplasmic extracts isolated from shake-tube mini-cultures (5 ml). Culture supernatants were concentrated 20-fold using an Ultrafree-15 Biomax-10 centrifugal filter device (Millipore, Bedford, MA, USA) and dialyzed into PBS. The periplasmic extracts from cell pellets were prepared as described previously (Kipriyanov *et al.*, 1996b). For each scFv variant, three independent expression cultures were used. The concentrations of functional recombinant antibody fragments were determined from the fluorostaining of Jurkat cells using samples of periplasmic preparations and concentrated culture medium by the interpolation of their mean fluorescence intensities on the standard curves obtained with purified scFv of known concentration. At least four dilutions of samples were used for calculations.

Molecular modeling

Modeling was performed using AbM (Oxford Molecular, Oxford, UK). The framework was built by homology using HyHEL-5 (Sheriff *et al.*, 1987) for the parent light chain and 36–71 (Strong *et al.*, 1991) for the heavy chain. The complementarity determining regions (CDR) L1, L2, L3, H1 and H2 were built using canonical classes as proposed by Chothia *et al.* (1989) (CDR-L1 = Class 1, CDR-L2 = Class 1, CDR-L3 = Class 1, CDR-H1 = Class 1, CDR-H2 = Class 2) while CDR-H3 was built using the CAMAL algorithm (Martin *et al.*, 1989).

AbM sometimes has problems with junction regions where loops are spliced on to the framework. This can result in trigonal planar or D-amino acids at these junction sites. This occurred for residue H102 and this residue was rebuilt manually as an L-amino acid.

Other methods

Protein concentrations were determined by the Bradford dye-binding assay (Bradford, 1976) using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany). The concentrations of purified scFv were calculated from the A_{280} values using the extinction coefficient $\varepsilon^{1\ mg/ml} = 1.84$ derived from the Trp, Tyr and Phe content of the molecule using DNAid+1.8 Sequence Editor for Macintosh (F.Dardel and P.Bensoussan, Laboratoire de Biochimie, Ecole Polytechnique, Palaiseau, France). Analytical gel filtration of the scFv preparation was performed in PBS using a Superdex 75 HR10/30 column (Pharmacia). The sample volume and flow rate were 200 µl and 0.5 ml/min, respectively. For calibration of the column, a Low Molecular Weight Gel Filtration Calibration Kit (Pharmacia) was used.

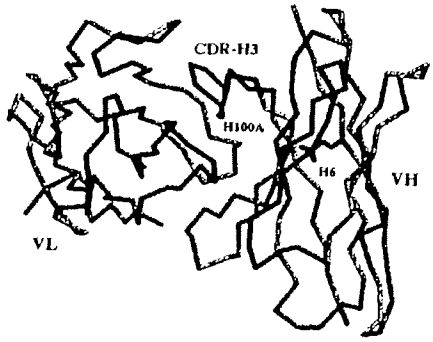

Fig. 1. Critical features of the OKT3 antigen binding site. The molecular model of OKT3 Fv is shown as a Cα trace with side chains of amino acid residues H100A and H6.

Results

Modification of PCR amplified OKT3 V_H gene

The V region genes of the murine monoclonal antibody OKT3 (Van Wauwe *et al.*, 1980) were amplified by PCR from a cDNA preparation using two pairs of highly degenerate primers. Ten clones of each amplified V_H and V_L gene were sequenced and found to be identical. All the analyzed heavy chain variable regions contained QVQLQE as the N-terminal sequence. This region was encoded by the 5′ primer Bi3f that contained a degenerate codon corresponding either to glutamic acid or glutamine residue at position 6 (Gotter *et al.*, 1995; Kipriyanov *et al.*, 1996b). The OKT3 scFv gene was assembled in the plasmid pOPE51 (Kipriyanov *et al.*, 1994) and expressed in *E.coli*. The resulting recombinant scFv product contained an unpaired cysteine residue near to its C-terminus that was specially introduced for making bivalent antibodies by chemical conjugation or for site-specific biotinylation (Kipriyanov *et al.*, 1994).

FACScan analysis demonstrated no binding of scFv-OKT3 isolated from periplasmic inclusion bodies (Kipriyanov *et al.*, 1994; Kipriyanov *et al.*, 1995) to CD3-positive Jurkat cells (data not shown). A detailed analysis of the predicted structure based on the OKT3 V domain sequences allowed us to identify two amino acid residues in the V_H domain that might be critical for the activity of this recombinant antibody (Figure 1). First, a comparison with the OKT3 cDNA sequence (Adair *et al.*, 1994) showed that position 6 of FR1 was occupied by glutamine but not by glutamic acid as in the PCR amplification product. Furthermore, the consensus sequences of the Kabat database demonstrated that the cloned V_H gene fragment belongs to mouse immunoglobulin subgroup IIb (Kabat *et al.*, 1991), in which 92% of the members have Q in position 6. Second, the OKT3 V_H domain was found to contain a cysteine residue in the CDR-H3 which could interfere with folding by disrupting normal disulfide bonding, or might be oxidized during IMAC on an Ni column under denaturing conditions (Kipriyanov *et al.*, 1994). Therefore, we performed site-specific mutagenesis of the V_H gene to substitute E6 by Q and C100A [numbering scheme of Kabat *et al.* (1991)] by S. This double-mutant scFv-dmOKT3 (Q,S) demonstrated strong binding to CD3-positive Jurkat cells and no interaction with CD3-negative JOK-1 cells when purified from inclusion bodies (data not shown).

Construction and expression of anti-CD3 scFv mutants

To clarify how the amino acid changes described above contribute to the activity of the anti-CD3 scFv, we investigated four different scFv variants: a variant containing E6 and C100A that was amplified from hybridoma cDNA by PCR (E,C), a variant corresponding to the cDNA sequence published for OKT3 (Q,C; Adair *et al.*, 1994) and two variants containing Ser instead of Cys at V_H position 100A (E,S and Q,S).

To avoid working with inclusion bodies, which have to be refolded and to prevent vector-derived C-terminal unpaired cysteines from affecting the scFv properties (e.g. possible formation of an additional intramolecular disulfide bond with Cys-100A or scFv dimerization), we chose the plasmid pHOG21 for expressing the mutated scFv genes (Figure 2A). The bacterial pHOG21 expression vector was designed for the high-level production of soluble recombinant antibody fragments in *E.coli* (Kipriyanov *et al.*, 1996b). The antibody V_H fragment is preceded by a *pel*B leader sequence for secretion of recombinant antibody into the periplasmic space. The C-terminus of the V_H domain and N-terminus of the V_L domain are joined by a flexible 17 amino acid tag-linker that includes a tubulin epitope recognized by mAb YOL1/34 (Breitling *et al.*, 1991). A short peptide tag containing an epitope of the proto-oncogene *c-myc* recognized by mAb 9E10 (Evan *et al.*, 1985) is located at the C-terminus of the V_L domain followed by six histidine residues to facilitate the isolation of recombinant antibody fragments by IMAC. The sequence of the OKT3 derived scFv assembled in the plasmid pHOG21 is shown in Figure 2B with the mutations at amino acid positions 6 and 100A of the heavy chain indicated.

Recently, we showed that the addition of 0.4 M sucrose to the growth medium gives a 15–25-fold increase in the yield of soluble scFv for bacterial shake-tube cultures and an 80–150-fold increase for shake-flask cultures (Kipriyanov *et al.*, 1997). We also found that the scFv could be made to accumulate in the periplasm or be secreted into the medium by simply changing the incubation conditions and the concentration of the inducer. Therefore, to obtain higher yields of soluble anti-CD3 antibody fragments, we incubated induced *E.coli* cells in the presence of 0.4 M sucrose. Western blot analysis of cell pellets and periplasmic extracts of bacterial cultures expressing the four variants of OKT3 derived scFv demonstrated substantial differences in the ratio of soluble and total scFv (Figure 3). While the total amount of recombinant product found in the cell pellet seemed to be equal for all scFv variants, much less soluble scFv was found for variants containing Glu at position 6 (Figure 3, lanes 2 and 6). FACScan analysis demonstrated the specific binding of periplasmic extracts for all the anti-CD3 scFv variants to CD3-positive Jurkat cells, although the fluorescence intensity obtained for scFvs with E6 was significantly lower (Figure 4A).

Purification of anti-CD3 scFv variants

To clarify whether the difference in antigen binding activity of periplasmic extracts containing different scFv variants (Figure 4A) is due to the difference in affinity or merely reflects the production levels of soluble antibody fragment, we performed a large-scale isolation of scFv using shake-flask bacterial cultures in the presence of 0.4 M sucrose. Under these conditions, we previously found that most of the secreted scFv was released into the medium (Kipriyanov *et al.*, 1997). The supernatant and periplasmic content of the induced bacterial culture was concentrated and passed through an Ni^{2+}

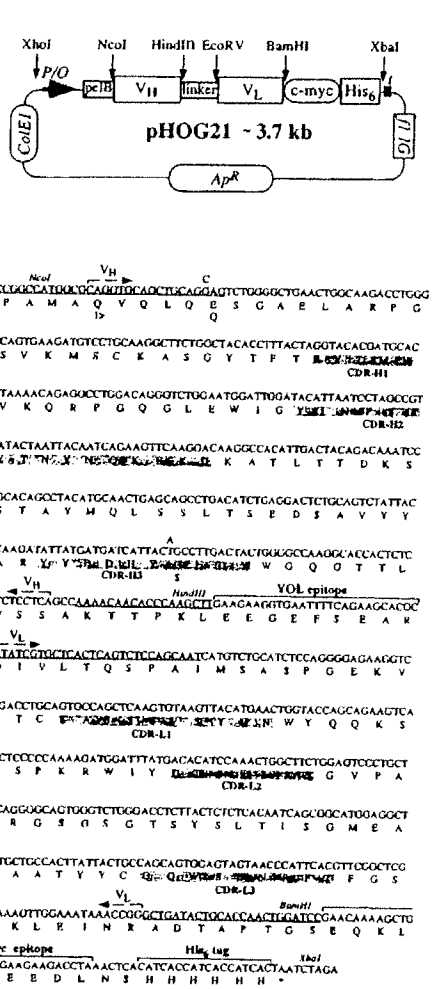

Fig. 2. The structure of scFv and expression vector. (A) Schematic representation of the plasmid pHOG21. Ap^R, ampicillin resistance-encoding gene; *c-myc*, a sequence encoding an epitope recognized by the monoclonal antibody 9E10; *ColE1*, origin of DNA replication; *f1IG*, intergenic region of phage f1; His_6, a sequence encoding six C-terminal histidine residues; linker, a sequence encoding 17 amino acids connecting the V_H and V_L domains; *pel*B, signal peptide sequence of bacterial pectate lyase; P/O, wt *lac* promoter/operator. (B) The nucleotide and deduced amino acid sequences of the scFv derived from hybridoma OKT3. The amino acid sequences corresponding to the complementarity determining regions (CDR) are shown shaded. The nucleotide sequences corresponding to PCR primers are underlined. The sequences coding for the YOL epitope in linker region as well as *c-myc* epitope and six histidines in the carboxy terminal part of the scFv are indicated.

charged Chelating Sepharose column. After washing the column with buffer containing 50 mM imidazole, the bound scFv was eluted with 250 mM imidazole as a single peak in 2.5 column volumes. This purification procedure allowed us to isolate scFv in one step with a purity of about 95% (Figure

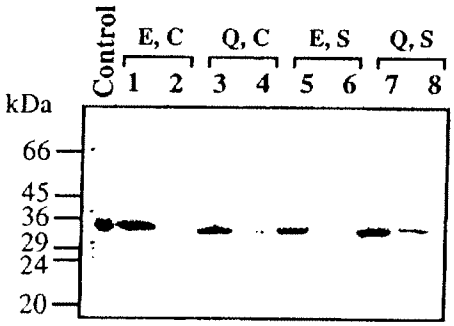

Fig. 3. Western blot analysis of cell pellets and periplasmic extracts from *E.coli* clones expressing different anti-CD3 scFv variants. Lanes: 1, 3, 5, 7, total cell lysate from induced bacteria corresponding to 100 µl of culture; 2, 4, 6, 8, periplasmic extracts corresponding to 180 µl of culture. The scFv were detected using mAb 9E10 recognizing the C-terminal *c-myc* epitope. As a control, 1 µg of pure scFv-dmOKT3 isolated from inclusion bodies was used. The positions of molecular mass markers are shown on the left.

5A). The main contaminant present in samples of scFv purified by IMAC has recently been identified as an *E.coli* metal-binding 27 kDa WHP protein (Wülfing *et al.*, 1994). An analysis of its amino acid composition showed that the WHP protein has an isoelectric point (*Ia*) of 5.16; anti-CD3 scFv variants were found to be more basic (the calculated *Ia* was between 7.27 for the E,C and 7.52 for the Q,S variant). This charge difference allowed us to purify the recombinant antibody fragments to homogeneity by ion-exchange chromatography on a Mono S column (Figure 5B). Analytical gel-filtration on a Superdex 75 column demonstrated that all the isolated scFv preparations consisted only of monomers (data not shown).

Affinity and stability measurements

Our attempts to use radioiodinated scFv preparations for measuring the direct binding of recombinant antibodies to CD3-positive Jurkat cells were unsuccessful. Unfortunately, iodination using chloramine-T yielded an inactive product for both anti-CD3 scFv and Fab fragment prepared from mAb OKT3 (data not shown). It is possible that iodination blocked tyrosine residues in the CDR regions that may be important for antigen-binding (Figure 2B). We therefore employed two different non-radioactive approaches based on flow cytometry (Bohn, 1980) that do not require any modification of the protein.

In the first approach, recombinant antibody fragments were incubated with cells as in a standard radioprotein binding assay, except that an anti-*c-myc* mAb and fluorescent anti-mouse IgG reagent were used to detect the amount of bound scFv. In comparison with a standard radioligand binding assay, the same variables (except the number of molecules bound at saturation) can be measured and an affinity constant determined from the slope of the resultant Scatchard curve (Chamow *et al.*, 1994).

The binding of scFv preparations was measured using human Jurkat cells as a source of naturally expressed cell bound CD3ε. Binding to CD3-negative JOK-1 cells was used as a negative control. The results of fluorostaining of Jurkat cells displayed in Figure 4B demonstrate that the same concentrations of different scFv variants yield similar fluorescence (slightly higher values were obtained for variants containing Gln at position 6). A pattern of increased fluorescence with

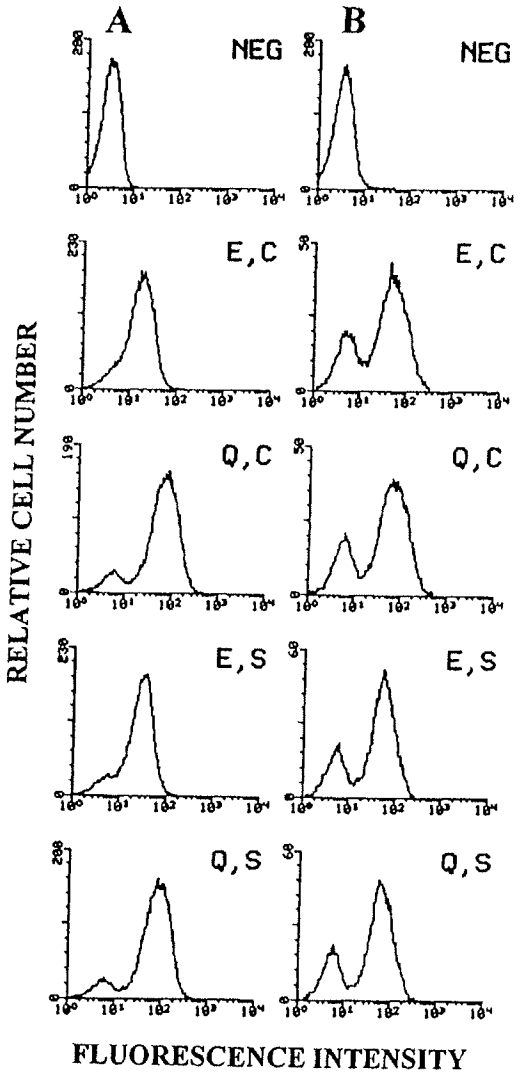

Fig. 4. Flow cytometric analysis of the binding of anti-CD3 scFvs to Jurkat cells. (A) Analysis of binding of periplasmic extracts. (B) Analysis of binding of pure scFv preparations at concentration 25 µg/ml. The presence of two peaks of fluorescence indicates that not all cells of the used line express CD3 antigen. As a negative control, binding to CD3-negative JOK-1 was used.

increased amounts of scFv was observed that seems to reach a plateau at higher concentrations (Figure 6A). On the basis of fluorescence measurements at different concentrations of added scFv, typical Scatchard curves were generated from which K_a values were derived (data not shown).

In a second approach, the binding efficiency of anti-CD3 scFv variants to Jurkat T cells was investigated by competition

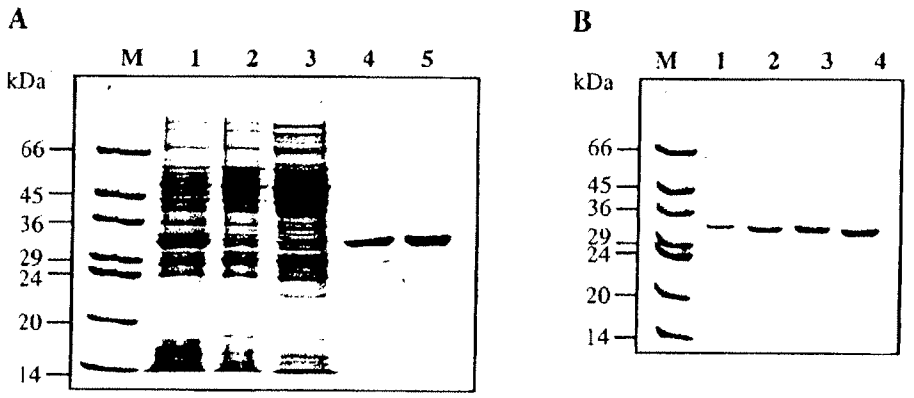

Fig. 5. 12% SDS–PAGE analysis of scFv preparations. (A) Analysis of scFv-dmOKT3 (Q,S) at different steps of purification. Lanes: M, molecular mass markers (values in kDa are shown on the left); 1, total cell lysate; 2, soluble periplasmic content; 3, concentrated culture medium; 4, scFv isolated by IMAC; 5, scFv purified by ion-exchange chromatography. (B) Analysis of purified scFv preparations for variant E,C (lane 1), Q,C (lane 2), E,S (lane 3) and Q,S (lane 4). The gels were stained with Coomassie Brilliant Blue.

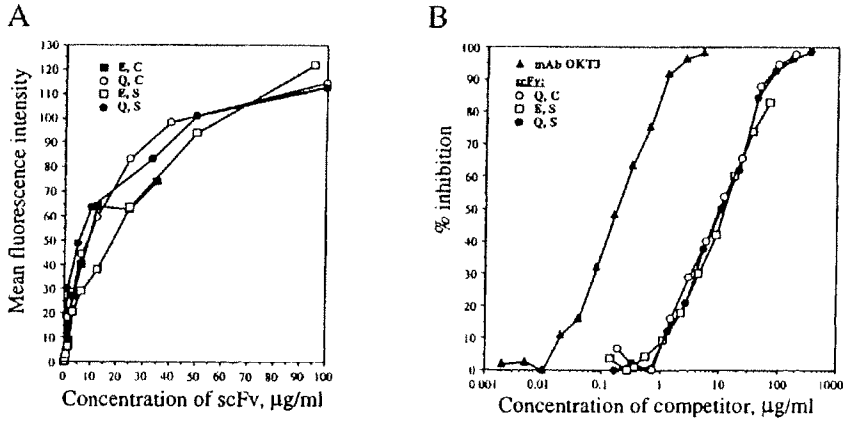

Fig. 6. Analyses of apparent affinities of scFv variants by flow cytometry. (A) Direct binding of different scFv variants to Jurkat cells. The cells were incubated with various concentrations of OKT3 derived scFv variants: E,C (filled squares), Q,C (open circles), E,S (open squares) and Q,S (filled circles). The curves were constructed by plotting the mean fluorescence intensity against the scFv concentration. (B) Inhibition of binding of FITC-OKT3 to Jurkat cells in presence of mAb OKT3 (filled triangles) and scFv variants: Q,C (open circles), E,S (open squares) and Q,S (filled circles).

with FITC-labeled mAb OKT3. The data presented in Figure 6B demonstrate that the OKT3 derived scFv Q,C, E,S and Q,S variants competed similarly, all at ~100 times the concentration of the intact IgG OKT3.

Analysis of the stability of anti-CD3 scFv variants after storage in PBS for 1 month at 4°C demonstrated a substantial loss of antigen-binding activity for scFv containing Cys in CDR-H3 (Figure 7).

Table I summarizes the results of the affinity and stability measurements. The apparent affinity values obtained for all the scFv variants proved to be quite close, indicating (i) only a slight effect of the sixth amino acid on the antigen binding and (ii) that the replacement of Ser for Cys in the middle of CDR-H3 does not disturb the antigen–antibody complex. Both the glutamic acid at position H6 and especially the cysteine at position of H100A led to a decreased stability of the scFv, probably because of a higher tendency for such antibody fragments to aggregate and/or for oxidation of unpaired cysteine residues during storage. No proteolytic degradation during storage was detected for any of the examined scFv variants (data not shown).

Analysis of expression yields

To study the influence of positions H6 and H100A on the production levels of soluble scFv fragments, we analyzed the antigen binding activities of periplasmic extracts and the concentrated culture medium of bacteria expressing scFv E,C, Q,C, E,S and Q,S variants. The expression yield data presented in Table I demonstrate that a single amino acid substitution of E6 by Q yields more than a 30-fold increase in soluble scFv product. In contrast, the single exchange of C100A by S led to a more moderate twofold increase in soluble scFv. These

effects were cumulative: the total yield of the Q,S variant was 66-fold higher than that for the E,C scFv variant (Table I). For all the examined variants, a small proportion of the functional soluble scFv was found to be released into the culture medium.

Discussion

Recombinant antibody fragments directed against cell surface antigens can provide useful components for the development of therapeutic agents. To target cytotoxic effector T cells to a tumor site, we have constructed an anti-human CD3 single-chain antibody by PCR amplification of the immunoglobulin variable domain genes from cDNA of the hybridoma OKT3. Expression of the assembled scFv gene in *E.coli* yielded a non-functional product after refolding from inclusion bodies.

In general, the primers we and other workers use for amplifying V genes from hybridoma cDNAs are designed to match all the known sequences of immunoglobulin genes. However, PCR amplification using degenerate primers does not always yield a gene with naturally occurring codons in the primer region (McCartney *et al.*, 1995). It is therefore often not possible to know which codons occur naturally if, as in our case, the DNA sequence was not then available. For example, the same set of primers resulted either in Glu or Gln in H6 after amplification of the V_H gene of an antibody against anti-human CD19 (Kipriyanov *et al.*, 1996b). Regarding the significance of this position, there was no indication in the literature that it may be critical for bacterially expressed antibody fragments.

To improve the properties of the recombinant antibody fragment, we focused on the amino acid residues which are structurally uncommon for the V_H subgroup IIb: glutamic acid at the position H6 of FR1 and a cysteine in the middle of the CDR-H3 loop. Site-specific mutagenesis and a change of expression system (soluble secreted scFv versus inclusion bodies) allowed us to clarify their influence on the production of a functional scFv antibody fragment.

We demonstrated that a single amino acid substitution of E by Q at position 6 of the heavy chain resulted in a 30-fold increase in soluble scFv product and significantly increased the stability of the recombinant molecule during storage. However, this substitution had very little effect on the affinity (scFv containing Q had affinity constants about 1.5 times higher than variants with E). This slight difference may be explained by the possible difference in the percentage of functional scFv (Kipriyanov *et al.*, 1994). We can therefore conclude that the sixth amino acid influences the correct folding of the V_H domain, perhaps by affecting some folding intermediate, but it has little or no effect on antigen binding. This conclusion was supported by computational molecular modeling. Examination of the residues which surround position 6 of the heavy chain in the three-dimensional model reveals no reason why a Glu or Gln residue should have any significant effect on the conformations of the CDRs (Figure 1).

It is not clear how Glu may effect the folding of the scFv fragment in the bacterial environment because very little is known about this process. Attempts have been made to prevent the side reaction of aggregation by overexpressing some known enzymes of the *E.coli* folding machinery such as the GroES/L chaperones, disulfide-isomerase and proline-*cis*–*trans*-isomerase (Knappik *et al.*, 1993; Duenas *et al.*, 1994). However, these proteins did not increase the yield of soluble antibody fragments. The presence of a periplasmic chaperone has therefore been postulated but not yet identified (Wülfing and Plückthun, 1994). From a variety of experiments, evidence is accumulating that the primary sequence of the antibody

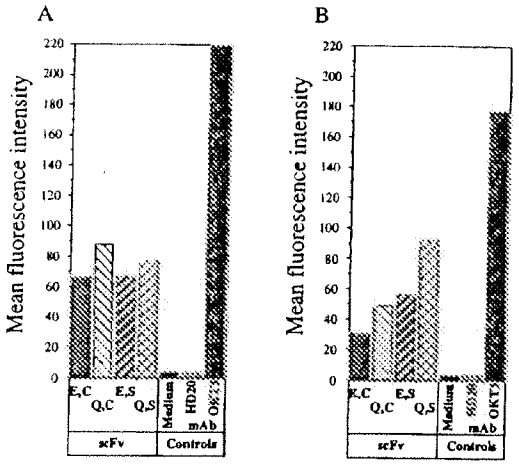

Fig. 7. Flow cytometric analyses of interaction of OKT3 derived scFv variants with Jurkat cells. (A) Fluorescence intensity obtained for fresh scFv preparations at concentration 25 μg/ml. (B) Fluorescence intensity obtained for the same scFv preparations after storage in PBS for 1 month at 4°C. As controls, the interaction of culture medium, mAb OKT3 and irrelevant mAb HD20 with Jurkat cells is shown.

Table I. Expression levels of anti-CD3 scFv variants, their stabilities and affinities to human CD3 antigen

scFv variant	Yield of scFv (μg/l of culture)[a]	Release of scFv (%)[b]	K_a[c] ($M^{-1}/10^7$)	K_a[d] ($M^{-1}/10^7$)	Stability (%)[e]
E,C	72.7 ± 19.5[f]	9.6 ± 1.4[f]	1.09	n.d.[g]	37.87
Q,C	2314.7 ± 578.8	24.4 ± 1.7	1.96	3.16	46.63
E,S	148.4 ± 37.7	25.4 ± 7.9	1.27	2.49	70.10
Q,S	4846.0 ± 477.3	29.4 ± 1.9	1.42	2.95	100

[a]Total amount of soluble scFv both in crude periplasmic extract and culture medium estimated by flow cytometry.
[b]Percentage of total scFv amount found in culture medium.
[c]Binding constants as determined by cytofluorometric Scatchard analysis.
[d]Binding constants as determined from a competitive inhibition assay using FITC-OKT3.
[e]Activity (%) after 1 month at 4°C as determined by flow cytometry.
[f]Arithmetic mean and standard deviation based on three independent experiments.
[g]Not determined.

plays a decisive role in the efficiency of folding in a bacterial environment (Carter *et al.*, 1992; Knappik and Plückthun, 1995). Our own results lead to a similar conclusion. The amino acid H6 influences not only the folding efficiency but also the stability of the correctly folded scFv.

The single exchange of Cys at position H100A by Ser also led to a twofold increase in soluble scFv. Three residues before the start of CDR-H3 is a conserved cysteine at position H92 which forms a structural disulfide bond with position H22. Thus, having another Cys nearby (at H100A) could easily allow mis-folding where H100A instead of H92 is involved in forming the disulfide bond with H22, thereby generating a mis-folded, insoluble and non-functional product. Analogously, Ostermeier *et al.* (1995) demonstrated that substitution of an uncommon cysteine at position H50 (first amino acid of CDR-H2) by a serine led to a 20-fold increase in soluble Fv production. Since the authors of this work were working with an Fv fragment, a mutation in the V_H domain can only influence the yield of the heavy chain fragment. Although direct comparisons of the influence of Cys residues in different CDRs on correct folding cannot be made, these results suggest that such uncommon residues may be more critical for the folding of a single antibody domain (V_H) than for scFv.

In our case, a cysteine was substituted that is present directly in the middle of CDR-H3, which is in the middle of the antigen-combining site and generally has the greatest influence on binding affinity. CDR-H3 plays a prominent role not only in ligand binding, but also in the contact with the V_L domain and with the other CDRs (Padlan, 1994). Although cysteine can occasionally form hydrogen bonds, this is rare in proteins (Baker and Hubbard, 1984) and it is a relatively hydrophobic residue. We therefore considered two possible mutations at H100A: serine (maintaining the size as closely as possible, but introducing a very hydrophilic residue) and valine (increasing the hydrophobic nature, but adding an extra atom). Given that the residue is exposed to solvent in the model, we chose to make the mutation to serine since any increase in hydrophobicity could lead to a change in folding of the loop. We were aware, however, that the substitution might interfere with antigen binding or influence the contact between the variable domains. Fortunately, the Cys to Ser mutation had no effect on antigen binding and, as hoped, led to a significant improvement in the stability of the scFv. Although the exposure patterns of the various amino acid types in immunoglobulins are comparable to those in other water-soluble proteins, cysteines are more exposed in CDRs than they are in the framework regions (Padlan, 1994). This is especially true for short (10 residues or less) hypervariable loops which do not have much opportunity to bury one of their residues while maintaining a distorted hairpin conformation for antigen binding. Exposure of the cysteine 100A SH group to solvent may result in oxidation or modification of the group over time and this may have an influence on the stability of the antigen–antibody complex. It is also possible that the unpaired cysteines of two adjacent scFv molecules could form a disulfide bond, thus giving rise to inactive and probably insoluble scFv dimers and causing a decrease in the concentration of functional scFv. These factors would explain the experimentally observed instability during the storage of scFv variants containing Cys in CDR-H3.

It is worth noting that in the present work we actually compared two different strategies of folding, i.e. *in vivo* and *in vitro*. The renaturation procedure, which has been used to refold several antibody fragments (Kipriyanov *et al.*, 1994; Gotter *et al.*, 1995) and a more complex scFv::streptavidin fusion protein (Kipriyanov *et al.*, 1996a) did not lead to the formation of an active scFv-E,C variant. These results point to limitations in the folding strategy *in vitro* compared with *in vivo* and indicate how such problems can be overcome.

In conclusion, we have constructed a modified version of an anti-human CD3 scFv antibody fragment with improved stability *in vitro* and increased production level in bacteria. This molecule may be particularly useful for the creation of recombinant cytotoxic immunoconjugates.

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